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13. ABSTRACT *(Maximum 200 Words)*

The idea that timing of surgery for breast cancer during a pre-menopausal woman's menstrual cycle can impact on survival has been a controversial one. Some clinical studies have shown improved survival when surgery was performed during the luteal phase of the cycle, while other studies failed to find this association. We previously showed that gene expression patterns varied in human breast tumors resected in different menstrual phases, and it is also known that normal tissues respond to cyclic hormones. Tumor cells shed at surgery at different menstrual phases thus might vary in their ability to form metastases, depending on hormone-responsive variations in host tissues and/or tumor cells. Our goal in this CONCEPT project was to begin to develop animal models to test this idea. To mimic possible shedding of tumor cells at surgery, we injected cells into mice intravenously during either proestrus or metestrus. Initially, as a control, we used a non-breast cancer cell line, murine B16F10 melanoma cells. We found unexpected differences in the organ specificity of metastases from these cells. Based on our initial success in developing this model system, we will continue and extend these studies in the future, to other cell lines and to mechanistic studies. Clarification of this phenomenon and its possible mechanisms has the potential to make an impact on survival from breast cancer.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
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INTRODUCTION

The idea that timing of surgery for breast cancer during specific phases of a pre-menopausal woman's menstrual cycle can impact on her survival has been a controversial one. Some clinical studies have shown improved survival when surgery was performed during the luteal phase of the cycle, while other studies failed to find this association. We previously showed that gene expression patterns varied in premenopausal women's breast tumors that were resected in different menstrual phases, and it also is known that normal tissues respond to cyclic hormones. Tumor cells shed at surgery at different menstrual phases thus might vary in their ability to form metastases, depending on hormone-responsive variations in host tissues and/or tumor cells. Our goal in this CONCEPT project was to begin to develop animal models to test this idea. To mimic shedding of tumor cells at surgery, we injected cells into mice intravenously during either proestrus or metestrus. Initially, as a control, we used a non-breast cancer cell line, murine B16F10 melanoma cells. We found unexpected differences in the organ specificity of metastases from these control cells. While there was no difference in metastatic burden in the lung, one-third of the mice injected in metestrus had prominent ovarian metastases while mice injected in proestrus had none. These novel findings suggest that a fluctuating hormonal milieu may differentially affect interactions of circulating tumor cells and secondary tissues in the establishment of metastases. These preliminary results provide an intriguing suggestion that the hormonal status at the time of entry of cancer cells into the blood stream can determine whether metastases form in specific organs. It is important that this phenomenon be studied further, to clarify the potential of relatively simple manipulations (scheduling surgery at defined menstrual phases, or hormone treatment at the time of surgery) to significantly improve the probability of survival for pre-menopausal women

with breast (and perhaps other) cancers. The successful results obtained from this CONCEPT award have led to a submitted grant proposal to continue this work, by comparing these findings with breast cancer cell lines, and assessing possible mechanisms.

BODY

Because this was a CONCEPT award, there were no explicitly stated Tasks, but rather an outline of the proposed experimental strategy of work to be done, which was presented in the original application. The Rationale initially presented for the work, as well as the proposed Experimental Strategy, are presented below, followed by a description of our Research Progress.

Rationale: The idea that timing of surgery for breast cancer during a pre-menopausal woman's menstrual cycle can impact on survival is controversial (1,2). Some studies showed improved survival when surgery was performed in the second half of the cycle, while other studies did not. However, a meta-analysis did find a significant effect on survival (3). This idea first came from an animal model and was then tested in human breast cancer. In 1988, murine mammary tumors were surgically removed at specific stages of the murine fertility cycle (defined by vaginal cytology) and the development of metastases was assessed (4). Resection of tumors during proestrus resulted in cure of the mice 2.5X more often than resection at other phases of the cycle. This idea was extended to patients in a small retrospective study of 41 pre-menopausal women: 10-year disease-free survival was 4X better in women whose tumors were resected in the early luteal phase of their cycle, vs. removal in the follicular phase, suggesting that timing of breast surgery during the menstrual cycle might have an impact on a woman's probability for survival.

Some clinical data have supported the validity of this strategy in breast cancer (1-3), and the effect has been seen in both estrogen receptor negative (ER-) and ER+ tumors (5). However, two factors have prevented general clinical use of this controversial approach. One concern is the variation in

the (retrospective) clinical data (1-3). Second is the lack of a mechanism to explain the effect and models in which to study this experimentally. We previously had conducted a clinical study to assess a possible mechanism (6). We tested the idea that breast tumor tissue cycles in its malignant properties over the menstrual cycle. Tumors from 32 pre-menopausal women with breast cancer were obtained at surgery, with menstrual cycle data and hormone blood monitoring. We assessed RNA levels of candidate genes associated with metastatic ability, and found significantly higher expression of two proteinases (Cathepsin L, MMP-9) and p53, in tumors resected during follicular/ periovulatory phases of the menstrual cycle vs. other phases. Similar trends were seen for other proteinases (MMP-2, cathepsin D), with an opposite trend for MMP inhibitors TIMP-1 and -2. That study showed that gene expression can cycle in breast tumors, and supports a possible mechanism to explain the clinical data. Cells shed during surgery thus might vary in their ability to establish metastases, for tumors resected at different times of the cycle.

Such studies using human tumor material are challenging to co-ordinate and experimental manipulation is difficult. Animal models are thus needed in which experimental questions can be answered. Development of such models is unlikely to be supported by traditional granting mechanisms, and the CONCEPT award mechanism was ideal for initiating this sort of high-risk developmental work.

Animal studies prior to the work reported here had only been conducted using the original murine model (4). However, this model has several drawbacks. The tumor cells can only be grown in vivo, and do not grow in tissue culture as a cell line, making experimental manipulation of the cells difficult. Second the cells have been implanted sub-cutaneously, and more recent work has

shown the biological importance of orthotopic (mammary fat pad) implantation (7). In addition, confirmation in other models is important.

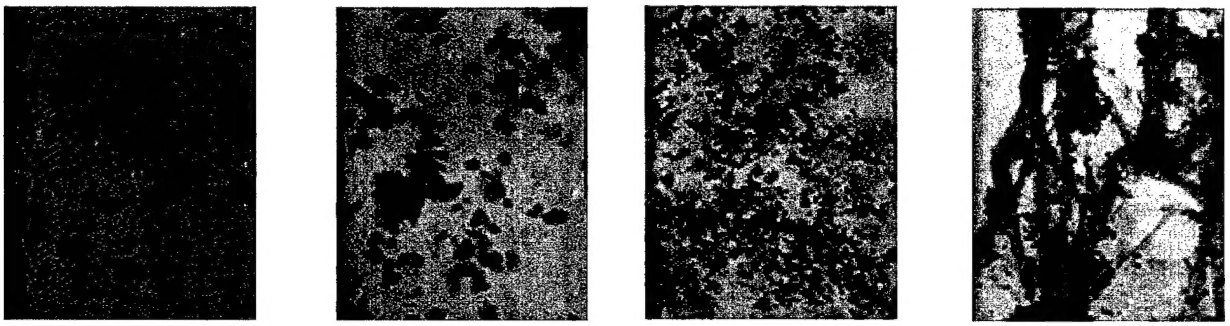
Experimental Approach: In the overall strategy that we outlined, we proposed to test murine and human breast cancer cell lines to learn if the effect can be reproduced in any of these models. We proposed to begin with metastatic MDA-MB-435 (human) and D2A1 (murine) cells, transfected to express Green Fluorescent Protein to facilitate detection of micrometastases. Both cell lines are ER-negative; we also will test ER-positive cell lines (e.g. MCF-7).

Cells will be implanted orthotopically in murine mammary fat pads to form primary tumors. We will then surgically resect tumors at defined phases of the estrous cycle, as determined precisely by vaginal cytology. (Because tumor implantation also requires surgery, we will compare surgical implantation at defined estrous phases). We also will inject cells intravenously in mice at different estrous phases, to mimic shedding of cells at the time of surgery; as noted below, we decided to start with this approach. End-points will be numbers and sizes of lung and other metastases.

We were aware that these experiments may be complex, with multiple variables. However, this CONCEPT award was designed to allow us to begin to assess what the important variables are, and to learn if any of the animal models we try can reproduce the phenomenon of variations in metastatic burden relative to phases of the estrous cycle (either at the time of surgical tumor removal or intravenous injection of the cells).

Research Progress: Our first step was to develop, in our lab, the procedures required to reliably determine the estrous phases of experimental mice. We have successfully established these techniques for routine use. The procedures are fairly straightforward, although they require skill and practice. We used daily vaginal cytology of the mice, as described by Rugh (8), taking samples 3-4 hours after light onset. Cellular contents of the vagina are rinsed out with sterile saline, the cells are stained with Toluidine Blue, and the estrous phase is determined by microscopy based on cell type. Photographs of representative cytology preparations are presented in Figure 1. Mice were categorized as being in proestrus, estrus, metestrus or diestrus based on cytology, and mice were monitored daily for at least one full estrous cycle (5-7 days) before being used for metastasis experiments.

Figure 1. Representative vaginal cytology from mice at various estrous stages.



PROESTRUS
Nucleated epithelial cells
Cornified epithelial cells
cells

ESTRUS
Cornified epithelial cells
No leukocytes

METESTRUS
Nucleated epithelial cells
Leukocytes

DIESTRUS
Mucosa
Nucleated epithelial

Leukocytes

Once we had established our ability to accurately stage the mice over the estrous cycle, our next goal was to carry out metastasis assays, in which cells would be injected in either proestrus or metestrus. These phases were chosen because they represent extremes of rising estrogen and decreasing progesterone (proestrus) vs. decreasing estrogen and increasing progesterone (metestrus). Our goal was to determine if any aspects of metastasis varied in mice injected at

different estrous phases, in order to begin to develop a mouse model to assess the effects of the estrous cycle on metastatic ability. We chose to begin the animal experiments with intravenously (i.v.) injected cells (tail vein), in order to mimic the shedding of tumor cells at the time of surgery, and as the most efficient way to obtain quantitative data. Cells injected by this route will initially be taken to the lung, where most will arrest; however, a small percentage of cells will pass through the lung microcirculation, through the heart, and will be distributed systemically via the arterial circulation (9). We also began with what we thought would be a negative control cell line, a hormone-independent, non-breast cancer cell line (i.e. one that we thought would be unlikely to give metastatic differences over the estrous cycle), in order to separate the role of tumor cell vs. host responsiveness to the hormonal status of the mouse. For this control, we chose the murine B16F10 melanoma cell line, which forms lung metastases following i.v. (tail vein) injection (10).

To our surprise, these experiments indicated that there were indeed differences in metastatic pattern in mice injected with the control cells at proestrus vs. metestrus. Because of this unexpected finding, the rest of our work on this award was focused on the B16F10 cell line, as we thought it important to understand this "control" experiment before proceeding to breast cancer cell lines.

Our findings from experimental metastasis assays with i.v. injection of B16F10 cells are summarized here. This experiment has been repeated five times, with the same conclusions, and data from the most recent experiment are presented. The estrous phase was determined for a series of C57Bl/6 female mice, using vaginal cytology as above. Mice identified as being in either proestrus or metestrus were injected with 5 X 10^4 B16F10 cells/mouse (i.v., tail vein). Mice were

sacrificed 24 days later and detailed autopsies were performed, to examine for metastases in lung and in extrapulmonary organs. The number of lung metastases did not differ in mice injected in the two phases (Figure 2).

However, there was a significant difference between the two groups in the incidence of metastases outside the lung. As shown in Table 1, none of the mice injected in proestrus had any ovarian metastases, while 31.6% of mice injected in metestrus had ovarian metastases, and many of these ovarian metastases were quite large (Figure 3). The difference in frequency of ovarian metastases was statistically significant between the proestrus and metestrus groups (p=0.0.36; Z-test), while frequency of metastases to other extrapulmonary sites did not differ. In addition, we found no correlation between the presence of extrapulmonary metastases and the metastatic burden in the lungs of individual mice, suggesting that the extrapulmonary metastases were not a result of secondary metastases from the lung.

We have thus demonstrated that the estrous phase of mice can influence the metastatic properties of cells injected intravenously in experimental metastasis assays. The goal of this CONCEPT award was to begin to establish animal models in which to study the timing of surgery effect, and our findings show that this assay can be used to study this phenomenon. We have recently initiated experiments to assess the mechanism(s) responsible for the differences in metastatic ability in cells injected at different estrous phases, by first asking if delivery of cells to extra-pulmonary sites varies with the estrous phase, or if equal numbers of cells are delivered to these sites and the primary effect of the estrous phase is to influence the growth of these cells. Furthermore, preliminary studies have recently been conducted with the ER- human breast cancer cell line,

MDA-MB-435, and have indicated that these cells also demonstrate the same estrous cycle-dependent, organ-specificity of metastasis as seen with the control B16F10 cells.

List of personnel receiving pay from this award:

Mr. Carl Postenka, registered medical laboratory technologist, with experience in experimental animal handling and metastasis research, was the only person paid from this award.

Figure 2. No difference in metastatic tumor burden in lung between Proestrus and Metestrus groups (N = 17-19 mice per group; p = 0.87), 24 days after tail vein injection of 5 X 10^4 cells/mouse.

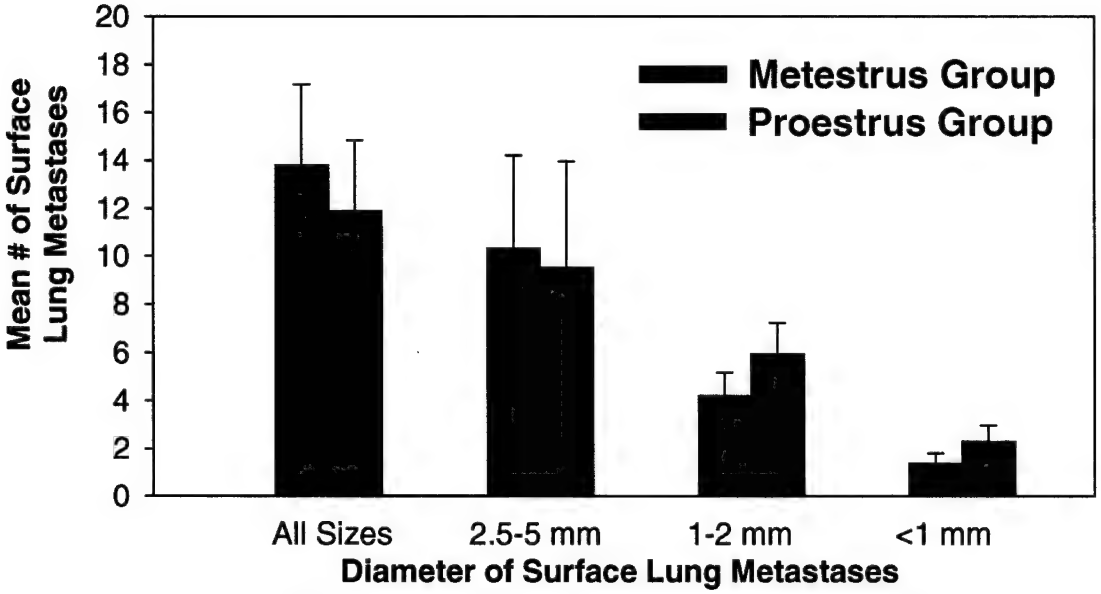
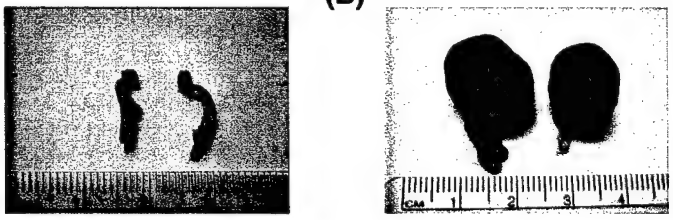


Table 1. Incidence of Extrapulmonary Metastases.

Site of Metastases	Proestrus Group	Metestrus Group	Significant difference between groups?
Spleen	5/17	2/19	P=0.313
Pancreas	1/17	1/19	P=0.517
Mesenteric Lymph Nodes	1/17	2/19	P=0.916
Abdominal Mesenteries	1/17	1/19	P=0.517
Ovaries	***0/17***	***6/19***	***P=0.036***

Figure 3. Representative ovaries from mice injected with B16F10 cells in either **(A)** protestrus or **(B)** metestrus. No ovarian metastases were detected in any ovaries of mice injected in proestrus, while 31.6% (6/19) of mice injected in metestrus had ovarian metastases, which were often quite large.

(A) **(B)**



KEY RESEARCH ACCOMPLISHMENTS

- We have established in the laboratory the procedures for determining the estrous phases of experimental mice, using daily vaginal cytology, and have sufficiently practiced these procedures such that mice can be reliably staged for phase of estrous cycle.

- We have successfully developed an animal model which shows differences in metastatic abilities in mice injected at distinct estrous phases. We first injected control B16F10 melanoma cells intravenously ("experimental metastasis assay") into mice at proestrus vs. metestrus, and have found unexpected and consistent evidence for differences in organ-specific metastasis with this control cell line: No mice injected in the proestrus phase had any extrapulmonary metastases, while one-third of mice injected in metestrus had extra-pulmonary, ovarian metastases.

- We thus have characterized a model in experimental mice which can be used to study the effect of the estrous cycle on aspects of the metastatic process.

REPORTABLE OUTCOMES

Abstracts presented:

Vantyghem SA, CO Postenka and AF Chambers. Estrus Cycle Influence on Organ-Specific Metastasis. Poster LB137, presented in the 'Late-Breaking Abstract' session at the American Association for Cancer Research annual meeting, San Francisco CA, April 9, 2002. **[enclosed as APPENDIX 1]**

Chambers, AF, SA Vantyghem and CO Postenka. Evidence that the Estrous Cycle Can Influence Organ-Specific Metastasis. Poster P25-4, presented at the Era of Hope Meeting for the Department of Defense Breast Cancer Research Program, Orlando FL, September 27, 2002. **[enclosed as APPENDIX 2]**

Funding applied for based on work supported by this award:

IDEA Proposal submitted to Department of Defense Fiscal Year 2002 Breast Cancer Research Program (submitted June 2002):

Proposal log number: BC021187

PI: Ann F. Chambers, Ph.D.

Title: Use of Animal Models to Study How Timing of Surgery During the Menstrual Cycle Can Affect Breast Cancer Metastasis and Survival

CONCLUSIONS

Our goal in these studies was to begin to develop animal models in which to test the controversial idea that timing of surgery during the menstrual cycle could affect survival from breast cancer. Our experiments with control cells have indicated that differences in experimental metastatic ability can indeed be detected in mice injected in two distinct phases of the estrous cycle. The availability of this model will now permit us to: (1) study estrogen receptor positive (ER+) and estrogen receptor negative (ER-) breast cancer cells, to determine the cell type specificity for these effects, and (2) to begin to assess mechanisms that could be responsible for the differences in organ-specific metastasis seen for cells injected into the circulation at different estrous phases.

The positive findings produced during the tenure of this CONCEPT award have led directly to the submission of an IDEA application to the Department of Defense Fiscal Year 2002 Breast Cancer Research Program, which would not have been possible without the CONCEPT support to allow us to develop this animal model. We are in the process of preparing a brief communication-type manuscript describing our initial findings, and we will hope that this publication will stimulate further research into this phenomenon and mechanisms that may contribute.

The availability of this animal model and the positive results we have obtained have indicated that the estrous phase of mice can effect metastatic outcome. These effects must now be assessed in various breast cancer cell lines, and the mechanisms responsible must be examined. If our current and future results can stimulate further research into this phenomenon and its mechanisms, it is our hope is that the resulting clarification of mechanisms will lead to appropriate translation to

patients, perhaps through identifying subsets of patients who would benefit by timed surgeries or pre-surgical hormonal interventions. Because these manipulations might have a major impact on survival of pre-menopausal women with breast cancer, with little or no patient toxicity, we believe that our successful development of an animal model in which to study the phenomenon is important, and we are committed to further work with this model to assess possible mechanisms.

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APPENDICES

Appendix 1: Abstract: Vantyghem SA, CO Postenka and AF Chambers. Estrus Cycle Influence on Organ-Specific Metastasis. Poster LB137, presented in the 'Late-Breaking Abstract' session at the American Association for Cancer Research annual meeting, San Francisco CA, April 9, 2002.

Appendix 2: Abstract: Chambers, AF, SA Vantyghem and CO Postenka. Evidence that the Estrous Cycle Can Influence Organ-Specific Metastasis. Poster P25-4, presented at the Era of Hope Meeting for the Department of Defense Breast Cancer Research Program, Orlando FL, September 27, 2002.

Abstract presented in the 'Late-Breaking Abstract' session at the American Association for Cancer Research annual meeting, April 9, 2002, San Francisco

Abstract Number: LB137

Estrus Cycle Influence on Organ-Specific Metastasis.

APPENDIX 1

Sharon A. Vantyghem, Carl O. Postenka and Ann F. Chambers, London Regional Cancer Centre, London, Ontario, Canada.

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The idea that survival of pre-menopausal breast cancer patients may be affected by the menstrual phase at the time of surgery remains controversial. Some retrospective clinical studies have shown improved survival when surgery was performed during the second half of the cycle (i.e. luteal phase), while other studies did not find this association. It is known that both normal and tumor tissues can respond to cyclical hormonal fluctuations with changes in gene expression. Thus, tumor cells shed during surgery, at different phases of the menstrual cycle, might vary in their ability to establish metastases. Additionally, secondary tissues might vary in their ability to support metastatic growth at different phases of the cycle. Our objective is to develop murine models to study this phenomenon and to investigate potential underlying mechanisms. While human and murine reproductive cycles differ in many respects, mouse proestrus corresponds to the follicular phase and metestrus to the luteal phase. Initially, to mimic the shedding of cells at the time of surgery, we injected tumor cells intravenously (tail vein) during either proestrus or metestrus (determined by vaginal cytology). Surprisingly, in a non-breast cancer control experiment in which B16F10 melanoma cells were injected into syngeneic mice, we found unexpected differences in the organ specificity of the metastases. While there was no difference between the two groups in metastatic tumor burden in the lung, 31.6% of the mice injected in metestrus (6/19) had prominent ovarian metastases while mice injected in proestrus (0/17) had none ($p = 0.036$). The presence of ovarian and other extrapulmonary metastases did not correlate with the metastatic burden in the lungs in either group, suggesting that the extrapulmonary metastases were not a result of secondary metastasis. These novel findings suggest that the fluctuating hormonal milieu of the host may differentially affect the interactions of circulating tumor cells and secondary tissues in the establishment of metastases.

EVIDENCE THAT THE ESTROUS CYCLE CAN INFLUENCE ORGAN-SPECIFIC METASTASIS

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The idea that timing of surgery for breast cancer during a pre-menopausal woman's menstrual cycle can impact on survival has been a controversial one. Some clinical studies have shown improved survival when surgery was performed during the luteal phase of the cycle, while other studies failed to find this association. We previously showed that gene expression patterns varied in breast tumors resected in different menstrual phases, and it also is known that normal tissues respond to cyclic hormones. Tumor cells shed at surgery at different menstrual phases thus might vary in their ability to form metastases, depending on hormone-responsive variations in host tissues and/or tumor cells. Our goal is to develop animal models in which to test this idea.

To mimic the shedding of tumor cells during surgery, we injected cells into mice intravenously (tail vein) during either proestrus or metestrus (determined by vaginal cytology). While human and murine reproductive cycles differ in many respects, mouse proestrus corresponds to the follicular phase and metestrus to the luteal phase. Initially, as a control, we used a non-breast cancer cell line, murine B16F10 melanoma cells. Surprisingly, we found unexpected differences in the organ specificity of metastases from these cells. While there was no difference in metastatic burden in the lung, 32% of mice injected in metestrus (6/19) had prominent ovarian metastases while mice injected in proestrus (0/17) had none ($p = 0.036$). The presence of ovarian or other extrapulmonary metastases did not correlate with the metastatic burden in the lungs in either group, suggesting that the extrapulmonary metastases were not the result of secondary metastasis.

These novel finding suggest that a fluctuating hormonal milieu may differentially affect interactions of circulating tumor cells and secondary tissues in the establishment of metastases. While further work is necessary to characterize this phenomenon, these preliminary results provide an intriguing suggestion that the hormonal status at the time of entry of cancer cells into the blood stream can determine whether metastases form in specific organs. It is important that this phenomenon be studied further, to clarify the potential of relatively simple manipulations (scheduling surgery at defined menstrual phases, or hormone treatment at the time of surgery) to significantly improve the probability of survival for pre-menopausal women with breast (and perhaps other) cancers.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0628 supported this work.